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研究課題名（和文）Identifying malaria vaccine candidate antigens using genetic linkage analyses

研究課題名（英文）Identifying malaria vaccine candidate antigens using genetic linkage analyses

研究代表者
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研究成果の概要（和文）：We have described here in detail the phenotypic, structural, copy number and nucleotide-level variations among several P. vinckei isolates. We hope that these efforts would greatly aid genetic linkage studies in the future to resolve genotype-phenotype relationships.

研究成果の学術的意義や社会的意義
Plasmodium vinckei isolates display a large degree of phenotypic and genotypic diversity and could serve as a resource to study parasite virulence and immunogenicity. Amenability to genetic crossing and transfection make them also suitable for classical and functional genetics to study malaria.

研究成果の概要（英文）：We have generated a comprehensive genetic resource for P. vinckei comprising of five reference-quality genomes, one for each of its subspecies, blood-stage RNA sequencing data for five P. vinckei isolates, and genotypes and growth phenotypes for ten isolates. Additionally, we sequenced seven isolates of the RMP species Plasmodium chabaudi and Plasmodium yoelii, thus extending genotypic information for four additional subspecies enabling a re-evaluation of the genotypic diversity and evolutionary history of RMPs. The subspecies from the highland forests of Katanga, P. v. vinckei, has a uniquely smaller genome, a reduced multigene family repertoire and is also amenable to transfection making it an ideal parasite for reverse genetics. We also show that P. vinckei parasites are amenable to genetic crosses.

研究分野：Parasitology

キーワード：Malaria　Genetics　Genomics

１．研究開始当初の背景

Malaria kills almost 400,000 young children every year. Despite significant financial investment and decades of research, we have no effective vaccine, and the drugs and anti-mosquito measures are failing. Recently, there have been reports that the malaria parasite is developing resistance against artemisinin in Africa (3); a major set-back in the fight against this disease. If resistance to this drug continues to spread, it is conceivable that the burden of malaria will increase further. Given this situation, new strategies to control malaria are urgently required.

２．研究の目的

New vaccine candidates for malaria, especially against the blood stages, are required to inform rational vaccine design. To identify diverse and numerous vaccine candidate antigens, it is necessary to utilize a model system with a large amount of inherent diversity. My lab currently houses **one of the largest collections of rodent malaria isolates in the world**. Included in this collection are 41 strains of P. vinckei, including multiple strains of 5 distinct sub-species. We have recently performed an extensive phenotypic, genotypic and transcriptomic characterization of 15 of these strains, including whole genome sequencing. This work has revealed extensive phenotypic and genetic polymorphism both within and between sub-species. Furthermore, preliminary studies have strongly suggested that immunity generated through exposure and drug cure of blood stages of these strains is strain-specific.

This project will aim to identify novel antigens in Plasmodium vinckei. Given the high degree of conserved genetics between the rodent and human malaria parasites, these will likely be of relevance for the design of malaria vaccines. Plasmodium vinckei offers a much higher degree of polymorphism within the clade than P. yoelii, increasing the chances of discovering novel antigen vaccine targets.

３．研究の方法

A total of seventeen RMP isolates were revived from frozen parasitized blood stabilates and propagated in female ICR and CBA mice. These included ten P. vinckei isolates consisting of one isolate of P. v. vinckei (PvvCY), two isolates each of P. v. brucechwatti (PvbDA and PvbDB), P. v. lentum (PvlDE and PvlDS) and P. v. petteri (PvpCR and PvpBS) and three isolates of P. v. subsp. (PvsEH, PvsEE and PvsEL). In addition to this, six P. yoelii isolates representing its four subspecies; three P. y. yoelii isolates (Pyy33X, PyyAR and PyyCN), one isolate each of P. y. nigeriensis (PynD), P. y. killicki (PykDG) and P. yoelii subsp. (PysEL); and one P. chabaudi subsp. isolate (PcsEF) were revived. The rodent malaria parasites isolated from Cameroon in 1974 by J. M. Bafort are currently without subspecies names, being designated as P. yoelii subsp., P. vinckei subsp. and P. chabaudi subsp.. We now present the full genome sequence data for these isolates and show they form distinct clades within their parent species. Therefore, we proposed the following subspecies names; Plasmodium yoelii cameronensis, from the country of origin; Plasmodium vinckei baforti, after J. M. Bafort, the original collector of this subspecies; and Plasmodium chabaudi esekanensis, from Eséka, Cameroon, the town from the outskirts of which it was originally collected. Eight uncloned stabilates (PvvCY, PvbDA, PvbDB, PvlDE, PvsEE, PyyCN, PynD and PykDG) were cloned by limiting dilution

and one clone each were chosen for subsequent experiments. We then carried out extensive phenotyping, genotyping, and immune selection characterisation on these strains.

4．研究成果

We followed the infection profiles of ten P. vinckei isolates in female CBA/J mice (five biological replicates per group) to study their virulence traitsP. vinckei parasites are morphologically indistinguishable from each other, prefer to invade mature erythrocytes, are largely synchronous during blood-stage growth and display a characteristically rich abundance of haemozoin crystals in their trophozoites and gametocytes (Fig. 1). Parasitaemia was determined daily to measure the growth rate of each isolate and host RBC density and weight were measured as indications of "virulence" (harm to the host) (Fig1). The P. v. vinckei isolate PvvCY was highly virulent and reached a parasitaemia of 89.4% ± 1.4 (standard error of mean; SEM) on day 6 post-inoculation of 1×10^6 blood-stage parasites intravenously, causing host mortality on that day. Both strains of P. v. brucechwatti, PvbDA and PvbDB were virulent and killed the host on day 7 or 8 post infection (peak parasitaemia of around 70%). The P. v. lentum parasites PvlDS and PvlDE were not lethal and were eventually cleared by the host immune system, with PvlDS's clearance more prolonged than that of PvlDE (parasitaemia clearance rates; PvlDS = 10%day^{-1}; SE = 1.1; p value of linear fit = 0.0025; PvlDE = 16.5%day^{-1}; SE = 3.9; p value = 0.023). The P. v. petteri isolates PvpCR and PvpBS reached peak parasitaemia along similar timelines (6–7 dpi), but PvpCR was virulent (peak parasitaemia = 60.4% ± 2.4 on day 6) and could sometimes kill the host while PvpBS maintained a mild infection. RBC densities reduced during the course of infection proportionally to the rise in parasitaemia in all the P. vinckei infection profiles studied. There were differences, however, in the patterns of host weight loss. Mild infections by P. v. lentum isolates (maximum weight loss in PvlDE = 0.4 mg ± 0.4 and PvlDS = 1.8 mg ± 0.4), P. v. petteri BS (0.6 mg ± 0.2) and P. v. baforti EE (1.7 mg ± 0.3) did not cause any significant weight loss in mice, whereas the virulent strains, P. v. petteri CR (4 mg ± 0.2) and P. v. brucechwatti isolates (PvbDA = 3.5 mg ± 0.4 and PvbDB = 2.1 mg ± 1.7), caused around a 20% decrease in weight. Virulent strains PvvCY (1.7 mg ± 0.2) and PvsEH (0.5 mg ± 0.1) did not cause any significant weight loss during their infection before host death occurred.

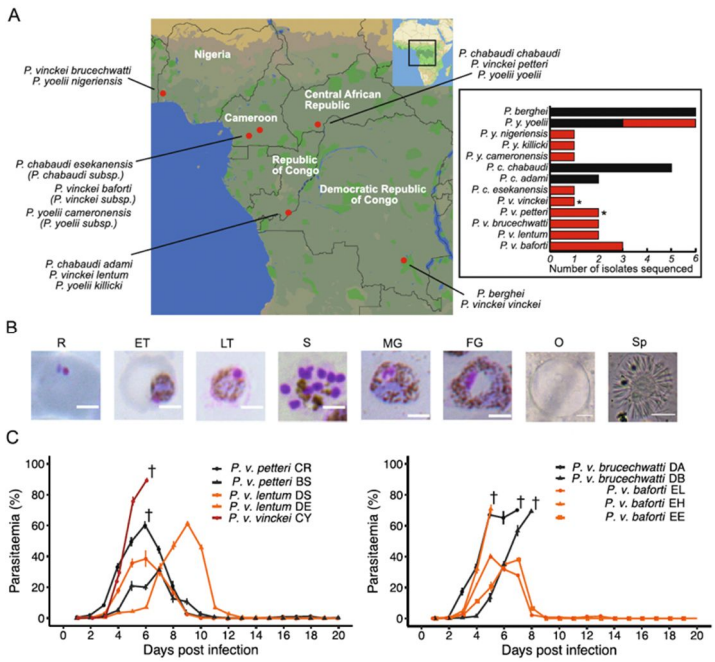

Figure 1 Plasmodium vinckei parasites and their phenotypic characteristics. **a** Rodent malaria parasite species and subspecies and the geographical sites in sub-Saharan Africa where from which they were isolated (modified from [1]). Plasmodium vinckei is the only RMP species to have been isolated from five different locations. Inset: To date, several RMP isolates have been sequenced (black) to aid research with rodent malaria models. Additional RMP isolates have been sequenced in this study (red) to cover all subspecies of P. vinckei and further subspecies of Plasmodium chabaudi and Plasmodium yoelii. **b**Morphology of different life stages of P. vinckei baforti EL. R: Ring, ET: early trophozoite, LT: Late trophozoite, S: Schizont, MG: Male gametocyte, FG: Female gametocyte, O: oocyst and Sp: Sporozoite. Plasmodium vinckei trophozoites and gametocytes are morphologically distinct from other RMPs due to their rich haemozoin content (brown pigment). **c**Parasitaemia of ten P. vinckei isolates (split into two graphs for clarity) during infections in mice (n = 5) for a 20-day duration. † denotes host mortality. Plasmodium vinckei isolates show significant diversity in their virulence phenotypes

Several exported proteins and surface antigens were identified to have undergone positive selection. PVVCY_0100120 (PCHAS_0100651 being the gene ortholog in P. chabaudi) has a circumsporozoite-related antigen PFAM domain (PF06589) and is a conserved protein found in all RMPs except P. berghei. PVVCY_1200100 (PBANKA_1002600) is a merozoite surface antigen, p41 that is secreted following invasion.

We then searched for the presence of any geographic location-specific selection pressures among the lowland forest isolates. Plasmodium vinckei isolates from Nigeria, Congo and Cameroon (P. v. brucechwatti, P. v. lentum and P. v. baforti respectively) were compared with P. v. petteri CR from the CAR. To see if similar selection pressures have acted on other RMP species too, we also analysed the P. yoelii (Nigerian PynD, PykDG from Congo and the Cameroonian PysEL versus Pyy17X from CAR) and P. chabaudi isolates (Cameroonian PcsEF versus PccAS from CAR) from the same regions.

Several exported and rhoptry-associated proteins were identified as having been subject to the influence of positive selection in each comparison but there was no overlap of positively selected genes among the lowland forest isolates. We identified a conserved rodent malaria protein of unknown function (PVVCY_0501990; PBANKA_051950) that seems to be under significant positive selection with high Ka/Ks values (ranging from 2.14 to 4.39) in all lowland P. vinckei comparisons except P. v. petteri–P. v. baforti. The P. yoelii ortholog of this protein was also positively selected among P. y. yoelii, P. y. nigeriensis and P. y. killicki but was not under selection within the P. y. yoelii isolates, signifying region-specific selection pressures.

Genetic crossing can be performed between P. vinckei isolates

The availability of several isolates within each P. vinckei subspecies with varying growth rates and wide genetic diversity makes them well-suited for genetic studies. Therefore, we attempted genetic crossing of the two P. vinckei baforti isolates, PvsEH and PvsEL, that displayed differences in their growth rates. Optimal transmission temperature and vector stages were initially characterised for P. v. baforti EE, EH and EL. Each isolate was inoculated into three CBA mice and on day 3 post infection, around 100 female A. stephensi mosquitoes were allowed to engorge on each mouse at different temperatures—21 °C, 23 °C and 26 °C. All three P. v. baforti isolates were able to establish infections in mosquitoes at 23 °C and 26 °C, producing at least 50 mature oocysts on day 15 post-feed but failed to transmit at 21 °C . Four to five oocysts of 12.5–17.5 μm diameter were observed at day 8 post-feed in the mosquito midgut and around a hundred mature oocysts of 50 μm diameter could be observed at day 15 post-feed. Some of these mature oocysts had progressed into sporozoites but only a very few (less than 10) appeared upon disruption of the salivary glands.

A genetic cross between PvsEH and PvsEL was performed. Both PvsEL-specific (11) and PvsEH-specific (17) markers were found in the 28 markers sequenced (one marker, PVSEL_0600390, could not be amplified). Also, five chromosomes clearly showed evidence of chromosomal cross-over since they contained markers from both isolates (see Fig. 3), thus confirming a successful P. vinckei genetic cross. However, all four clones had the same pattern of recombination which suggests that the diversity of recombinants in the cross-progeny was low and a single recombinant parasite might have undergone significant clonal expansion.

P. vinckei parasites are amenable to genetic manipulation

We asked if P. vinckei parasites can be genetically modified by applying existing transfection and genetic modification techniques routinely used in other RMPs. Plasmodium v. vinckei CY was chosen to test this because the isolate naturally established a synchronous infection in mice and reaches a high parasitaemia, which results in an abundance of schizonts for transfection. We aimed to produce a PvvCY line that constitutively expresses GFP-Luc (green fluorescent protein-firefly luciferase) fusion protein, similar to those produced in P. berghei and P. yoelii. A recombination plasmid, pPvvCY-Δp230p-gfpLuc, was constructed to target and replace the dispensable wildtype P230p locus in P. v. vinckei CY (PVVCY_0300700) with a gene cassette encoding for GFPLuc and a hdhfr selectable marker cassette (Fig. 3).

Transfection of purified PvvCY schizonts with 20 µg of linearized pPvvCY-Δp230p-gfpLuc plasmid by electroporation, followed by marker selection using pyrimethamine yielded pyrimethamine-resistant transfectant parasites (PvGFP-Luc$_{con}$) on day 6 after drug treatment. Stable transfectants were cloned by limiting dilution and plasmid integration in these clones was confirmed by PCR. Constitutive expression of GFPLuc in PvGFP-Luc$_{con}$ asexual and sexual blood-stage parasites was confirmed by fluorescence live cell imaging (Fig. 3). GFPLuc expression in PvGFP-Luc$_{con}$ oocysts was confirmed by fluorescence imaging of mosquito midguts 7 days after blood meal.

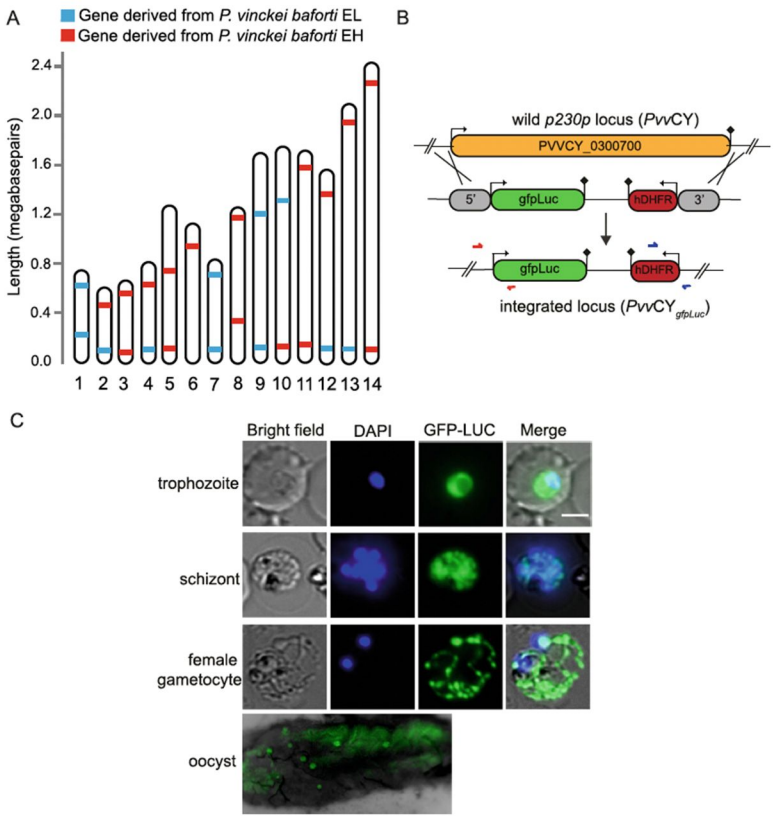

Figure 3. Phenotypic variation and genetics in Plasmodium vinckei parasites. **a** Schematic of isolate-specific genetic markers detected in clonal line of PvsEL X PvsEH cross-progeny by Sanger sequencing. Genetic markers from both EH (red) and EL (blue) isolates were detected in the crossed progeny proving successful genetic crossing. **b** Schematic of homologous recombination-mediated insertion of a gfp-luciferase cassette into the p230p locus in P. vinckei CY. **c** GFP expression in different blood stages of PvvCY and luciferase expression of PvvCY oocysts in mosquito midgut

５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　4件／うち国際共著　4件／うちオープンアクセス　4件）

１．著者名 Ramaprasad Abhinay、Klaus Severina、Douvropoulou Olga、Culleton Richard、Pain Arnab	４．巻 19
２．論文標題 Plasmodium vinckei genomes provide insights into the pan-genome and evolution of rodent malaria parasites	５．発行年 2021年
３．雑誌名 BMC Biology	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1186/s12915-021-00995-5	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Subudhi Amit K.、O'Donnell Aidan J.、Ramaprasad Abhinay、Abkallo Hussein M.、Kaushik Abhinav、Ansari Hifzur R.、Abdel-Haleem Alyaa M.、Ben Rached Fathia、Kaneko Osamu、Culleton Richard、Reece Sarah E.、Pain Arnab	４．巻 11
２．論文標題 Malaria parasites regulate intra-erythrocytic development duration via serpentine receptor 10 to coordinate with host rhythms	５．発行年 2020年
３．雑誌名 Nature Communications	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41467-020-16593-y	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Tang Jianxia、Templeton Thomas J.、Cao Jun、Culleton Richard	４．巻 10
２．論文標題 The Consequences of Mixed-Species Malaria Parasite Co-Infections in Mice and Mosquitoes for Disease Severity, Parasite Fitness, and Transmission Success	５．発行年 2020年
３．雑誌名 Frontiers in Immunology	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fimmu.2019.03072	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Jianxia Tang, Thomas J Templeton, Jun Cao, Richard Culleton	４．巻 10
２．論文標題 The Consequences of Mixed-Species Malaria Parasite Co-Infections in Mice and Mosquitoes for Disease Severity, Parasite Fitness, and Transmission Success	５．発行年 2020年
３．雑誌名 Frontiers in Immunology	６．最初と最後の頁 3072
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fimmu.2019.03072	査読の有無 有
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〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関